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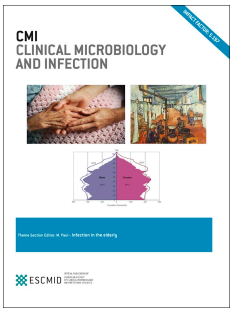

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Detection of the NS3 Q80K polymorphism by Sanger and deep sequencing in hepatitis C virus (HCV) genotype 1a strains in the United Kingdom

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ABSTRACT

The Q80K polymorphism in the HCV NS3 enzyme reduces susceptibility to simeprevir and other novel protease inhibitors. The study aimed to determine the prevalence of Q80K in treatment-naïve HCV-1a carriers in the North-West (NW) and South-East (SE) of England, investigate occurrence of Q80K as a minority variant, and characterise viral phylogeny. Plasma samples from subjects that were naïve to anti-HCV therapy underwent conventional (Sanger) and deep (Illumina-Miseq, 1% interpretative cut-off) sequencing of NS3. Q80K occurred in 44/238 subjects (18.5%, 95% confidence interval 13.6%-23.4%), including 19/70 (27.1%) in the NW and 25/168 (14.9%) in the SE (p=0.0425), with no difference by HCV RNA load or HIV status. **Q80K frequencies in reads of samples that underwent Illumina sequencing were >40% in all cases. Among subjects with Q80K, 5/44 (11.4%) showed one additional major resistance-associated mutation in NS3 detected at mutant frequency above (V36L, V55A) or below (V36M) 10%.** Phylogenetic analyses identified the two recognised HCV-1a lineages with (clade I) and without (clade II) Q80K. Overall, 148/238 (62.2%) sequences occurred within regional or inter-regional clusters, **each comprising 3-20 sequences**. There was no unique clustering of English sequences relative to strains from continental Europe and North America. In conclusion, Q80K was found at high prevalence among treatment-naïve HCV-1a carriers in England, and was reliably detected by conventional sequencing, **with no increased detection by deep sequencing.** English sequences were highly interspersed with sequences from elsewhere in Europe (clade II) and North America (clade I), and their phylogeny was consistent with multiple introductions from different areas.

INTRODUCTION

The HCV NS3 protease enzyme exhibits a high degree of genetic variability and only 47% of its amino acids are conserved among circulating HCV genotypes.[1] NS3 genetic heterogeneity and the associated molecular and structural differences influence HCV susceptibility to protease inhibitors (PIs), including licensed first-generation (telaprevir, boceprevir) and second-generation (simeprevir) compounds. HCV variants with reduced PI susceptibility have been observed in treatment-naive patients.[1-10] Among these, the glutamine to lysine substitution at codon 80 (Q80K) reduces susceptibility to simeprevir *in vitro* [2, 4, 11] and was also seen to reduce virological responses to simeprevir plus pegylated interferon alpha and ribavirin (P/R) in clinical studies.[12-14] Screening for Q80K is therefore recommended before starting simeprevir.[15]

The prevalence of Q80K among PI-naïve subjects varies by HCV genotype and subtype. An analysis of global NS3 sequences deposited in GenBank reported Q80K in 42% of HCV-1a strains, and also detected a high prevalence in HCV-5 and HCV-6 strains.[1] Among HCV-1a strains, prevalence of Q80K ranges between 3% and 47% by geographical location, exceeding 40% in North America.[2, 4] Reported prevalence is 20% overall across Europe, although differing markedly by country.[7-9] Most studies to date have produced prevalence estimates using conventional (Sanger) sequencing, which allows detection of virus variants present at a frequency ≥20% within a patient's sample.

This study had three aims. Firstly, to determine the prevalence of Q80K in NS3 sequences obtained from HCV-1a carriers attending for care in two regions of England in the United Kingdom(UK). A further aim was to investigate the occurrence of Q80K as a low frequency variant by deep sequencing, and thereby both gain insights into the viral quasispecies and determine whether conventional sequencing offers sufficient sensitivity for screening simeprevir candidates for the presence of Q80K. The third aim was to determine the phylogeny of NS3 sequences in relation to publically available sequences from the rest of Europe and North America.

METHODS

Study population

The study was performed retrospectively using stored plasma samples from consecutive adults that in 2006-2014 attended for care at the Royal Liverpool University Hospital in Liverpool (North-West region) and at King's College Hospital, Chelsea & Westminster Hospital, and Charing Cross Hospital in London (South-East region). Eligible patients were infected with HCV-1a **based on the genotyping method available at the local National Health System diagnostic laboratory; the assignment was confirmed by analysis of the NS3 sequences produced in this study.** Patients were naïve to all anti-HCV therapy. HIV status and HCV RNA load were retrieved from the clinics' databases. Ethics permission was granted by the South Berkshire Regional Ethics Committee to conduct the study after removing personal identifiers from the samples.

Sanger sequencing

HCV RNA was extracted with Nuclisens easyMAG (bioMérieux, Netherlands) and cDNA synthesis was performed with the Qiagen OneStep RT-PCR Kit (Hilden, Germany) with forward primer 3278 [GGAGACCAAGMTCATCACSTGG] and reverse primer 4032 [GCTCTTRCCGCTGCCRGTGGG]. The first-round amplicon was subjected to nested PCR using the Qiagen HotStarTaq Kit with forward primer 3307 [ACACCGCSGCGTGYGGKGACAT] and reverse primer 4014 [GGRGCRTGYAGRTGGGCCAC]. The second-round 727bp amplicon (aa180 of NS2 to aa204 of NS3) was purified with the Qiagen QIAquick PCR Purification Kit and sequenced using the BigDye Terminator Cycle Sequencing Kit v3.1 on the ABI Prism 3730 Genetic Analyser (Applied Biosystems®, USA). Consensus sequences were assembled using SeqScape (v2.7) and analysed with the geno2pheno system (http://hcv.geno2pheno.org/index.php; March 2015 version) for the presence of HCV resistance-associated mutations (RAMs).

Deep sequencing

HCV RNA was extracted with QiasymphonySP (Qiagen) using the Qiagen DSPVirus/Pathogen Midi Kit and the CellFree_500_V4 protocol; cDNA was produced using the ImProm II Reverse Transcription System (Promega, USA) and random primers. NS3 was amplified using Q5 High-Fidelity DNA Polymerase (New England Biolabs, USA) and the above primers. PCR products were purified using AMPure XP Beads (Beckman Coulter, USA) before quantification with the Quant-iT dsDNA High Sensitivity Kit (Life Technologies, USA)

on a Qubit 2.0 Fluorometer (Life Technologies). The Nextera XT Sample Preparation Kit (Illumina, USA) was used for library preparation and pooled amplicon libraries were sequenced on Illumina MiSeq using v2 reagents. Data generation and initial analysis were carried out at the Centre for Genomic Research at the University of Liverpool. Adapter sequences were trimmed from the Illumina reads using Cutadapt v1.2.1[16]; further trimming was done using Sickle v1.2 (http://github.com/najoshi/sickle) applying a minimum window quality score of 20. BAMStats (http://sourceforge.net/projects/bamstats/files/) was used to calculate the read statistics. Variants were analysed using the VirVarSeq pipeline,[17] which utilises the quality of the run and individual bases to filter poor quality bases and reduce false positive rates. A 727bp region of a HCV-1a molecular clone encoding full-length NS3 was amplified and sequenced in four replicates in order to estimate the assay error rate. The plasmid control yielded an average of 174,799 reads, with a mean coverage of 46,268 reads per nucleotide. The error rate, calculated by counting **false substitution reads** at the codon level of the molecular clone [18], was mean 0.6% (SD 0.2%) over NS3 amino acids 1-181.

Phylogenetic analysis

Reference HCV-1a sequences and all available HCV-1a NS3 sequences from North America and Europe with confirmed genotype assignment and country of origin were retrieved from the Los Alamos HCV database (hcv.lanl.gov/content/sequence/HCV/ToolsOutline.html; accessed on 8 March 2015) **(supplementary file). All sequences, including those produced in this study (Sanger sequences and NGS consensus sequences with 1% interpretative cut-off),** were aligned pairwise against the HCV-1a reference genome H77 (accession no. NC_004102) using MEGA v.6. The alignment **was trimmed** to codon positions 1-194 of NS3 (nucleotides 3421-4014 of H77). Sequences with gaps or ambiguous nucleotides affecting ≥50% of the targeted region or where codon 80 was missing or ambiguous were removed **(n=3)**, yielding a total of 1162 NS3 sequences for analysis. Two maximum-likelihood (ML) phylogenies were estimated with and without the 80 codon, using a general-time reversible nucleotide substitution model with gamma-distributed among-site rate variation and the ML method as implemented in PHYML v.3.0.[19, 20] Phylogenetic support was evaluated using a bootstrap approach and a total of 1000 bootstrap replicates were generated using the FastTree v.2.1.7 software;[21] local branch support was calculated by the Shimodaira–Hasegawa-like (SH-like) test. Phylogenies were visualized and annotated in FigTree (http://tree.bio.ed.ac.uk). Clusters (≥3 sequences) were identified by a bootstrap support >75% and were defined as regional if

comprising sequences unique to either the NW or the SE, and inter-regional if including sequences from both regions.

Statistical analysis

Descriptive statistics were used to analyse the prevalence of mutants in the study population (as the proportion of subjects showing the mutation) and the frequency of mutants in each patient's sample (as the proportion of deep sequencing reads showing the mutation). Proportion of subjects with Q80K by geographic location and HIV status were compared by Fisher's exact test. The Mann-Whitney U test was used to compare the HCV RNA load of patients with and without Q80K. The analysis was performed using SPSS v.21.

RESULTS

Study population

Overall 238 adults (median age 44 years, with no difference by region) infected with HCV-1a were studied, including 70/238 (29.4%) in the North-West (NW) and 168/238 (70.6%) in the South-East (SE). At the time of testing, HCV RNA load was median 6.3 $\log_{10}$ IU/ml (interquartile range,IQR 5.8-6.8), without differences between the two regions. Subjects were naïve to all anti-HCV therapy. A total of 61/238 (25.6%) subjects, all from the SE, were co-infected with HIV.

Q80K prevalence by Sanger sequencing

Overall, Q80K was detected in 44/238 subjects, yielding a prevalence of 18.5% (95% confidence interval, CI 13.6%-23.4%)(Table 1). Samples from two of the 44 subjects had a mixed first base at codon 80 (Q80Q/K). **The median HCV RNA load was 6.4 (IQR 5.8-6.7) vs. 6.3 (IQR 5.8-6.8) $\log_{10}$ IU/ml in samples with vs. samples without Q80K, respectively (p=0.63)**. The prevalence of Q80K was 19/70 (27.1%) in the NW and 25/168 (14.9%) in the SE (p=0.043). When comparing results by HIV status in the SE, Q80K prevalence was 18/107 (16.8.%) in HCV mono-infected subjects and 7/61 (11.5%) in HCV/HIV co-infected subjects (p=0.379). Table 1 shows other major and minor NS3 RAMs detected by Sanger sequencing.

Q80K prevalence by deep sequencing

A total of 178/238 (74.8%) samples underwent deep sequencing, comprising 28/178 (15.7%) with and 150/178 (84.3%) without Q80K by Sanger sequencing. An average of 58,585 reads per sample was obtained (median 53,413; IQR 40,740-70,255) with an average coverage per

nucleotide of 16,107. Prevalence of Q80K was 27/178 (15.2% 95% CI 9.9%-20.5%). All 28 samples showing Q80K by Sanger sequencing also showed the mutation by deep sequencing, usually (26/28 samples) with mutant frequencies of ≥98%. Of the two samples showing Q80Q/K by Sanger sequencing, one had a Q:K ratio of 54:46, and the other showed Q, K and L at a ratio of 57:41:2. Of 150 samples lacking Q80K by Sanger sequencing, none showed the mutation by deep sequencing when applying an interpretative cut-off of ≥1% for the frequency of mutants within the reads, as usually recommended.[22] Prevalence of Q80K increased when the interpretative cut-off was lowered, and was 3/150 (2%) for Q80K occurring at a frequency between ≥0.5% and <1%, and 15/150 (10%) at a frequency ≥0.2% and <0.5%. These cut-offs however fell within the estimated error rate of the assay (0.6%, SD 0.2%).

Deep sequencing (interpretative cut-off ≥1%) identified additional NS3 RAMs that had not been observed by Sanger sequencing and which occurred at frequencies between 1% and 6% (Table 1). **Among the 44 subjects with Q80K, 4/44 (9.1%) had one additional major NS3 RAM at frequency >10% including V36L in 3/44 (6.8%) and V55A in 1/44 (2.3%). In addition 1/44 (2.3%) subjects had V36M detected only by Illumina at a frequency of 3%**.

Phylogeny of NS3 sequences

HCV-1a strains separated into the two recognised distinct lineages with and without Q80K.[23, 24] The sequences harbouring Q80K at frequency <1% did not align with the Q80K lineage (data not shown). There was no unique clustering of the study English sequences (Figure 1), rather the sequences were interspersed with sequences from the rest of Europe and North America, and the phylogeny was consistent with multiple introductions from different areas. With the English sequences, four NW, eight SE, and 11 inter-regional clusters were identified, each consisting of 3-20 sequences (Figure 2). In total, 148/238 (62.2%) sequences (49/70, 70.0% in the NW and 99/168, 58.9% in the SE) were circulating as regional or inter-regional clusters.

DISCUSSION

This study detected the NS3 polymorphism Q80K at high prevalence among treatment-naïve HCV-1a carriers attending for care in England, particularly in the North-West where prevalence reached 27.1%. Previously published data from the UK include a small cohort of 38 HIV-positive patients with acute HCV-1a infection, of which 16% had Q80K.[6] A larger study analysed 159 subjects with HCV-1a that were enrolled in Phase II/III studies of

telaprevir and simeprevir, of which 23% showed Q80K[8]; these sequences are not available for analysis however, as to date they have not been deposited in the GenBank or the Los Alamos database. These data indicate that HCV-1a strains circulating in the UK have the highest prevalence of Q80K observed in Europe.

In this study the prevalence of Q80K varied by geographical region, while showing no difference by HIV status. Prevalence rates also show marked geographical variability in the rest of Europe.[7-9] The variable prevalence has not been linked to race or ethnicity [8], but rather is consistent with the circulation of two distinct HCV-1a lineages with and without Q80K.[23, 24] The Q80K-carrying lineage is believed to have originated in the United States in the 1940s.[23] In this study, the maximum likelihood phylogenetic analysis confirmed the two HCV-1a lineages, and detected multiple introductions of the two lineages in the UK, with wide interspersing of the English strains with other European and North American strains. Most HCV-1a sequences occurred as regional or mixed regional clusters, however, suggesting that for this cohort the majority of transmission events occurred within the UK.

The deep sequencing analysis showed that Q80K mutants occurred at high frequency (>40%) in the patients' samples, allowing detection by conventional sequencing in all cases. This finding is consistent with the observation that Q80K mutants have replication capacity similar to that of wild-type virus, which allows transmissibility and persistence at high frequency within the viral quasispecies despite the absence of drug selective pressure. There were no samples showing Q80K at a frequency below the detection threshold of Sanger sequencing (≥10-20%) and above the typical ≥1% interpretative cut-off for deep sequencing, which is recommended to differentiate biologically meaningful mutations from those caused by methodological errors.[22] When applying less stringent cut-offs (0.5% or 0.2%) the proportion of samples showing Q80K by deep sequencing increased. A previous study similarly found that among 21 subjects with Q80K detected by deep sequencing, four showed the mutation at a frequency <1%.[10] The estimated error rate of the assay (0.6%) and the observation that sequences showing Q80K at a frequency <1% did not align within the Q80K lineage suggest that the detection of Q80K at frequency <1% was spurious. **Furthermore, it remains to be determined whether any Q80K variant occurring at low frequency has clinical relevance.**

Q80K reduces HCV-1a susceptibility to simeprevir by 8-11 fold *in vitro*.[4, 8, 11] In HCV-1a infection, sustained virological responses (SVR) to combination therapy with simeprevir plus P/R are reduced by the presence of baseline Q80K in both treatment-naïve subjects (from 84% to 58%) and prior relapsers (from 79% to 47%).[11-13] The impact of Q80K on the combination of simeprevir plus sofosbuvir was not immediately apparent in some reported studies.[25] **However, a phase III, open-label, single-arm study with simeprevir plus sofosbuvir in cirrhotic patients with HCV-1 recently reported that SVR rates were higher in subjects without Q80K (92%) versus those with the mutation (74%).[26]** Q80K has no effects on susceptibility to telaprevir and boceprevir [4], but may have an effect on responses to asunaprevir.[27] Q80K also confers small reductions (~3-fold) in susceptibility to faldaprevir and paritaprevir *in vitro*, without necessarily affecting response rates.[28] The Q80L and Q80R mutations were detected in some subjects, usually at low frequency. Their significance is unclear. *In vitro*, the mutations confer 2-fold and 7-fold reductions in susceptibility to simeprevir, respectively.[11] Q80R has also been observed in a small number of patients experiencing failure of simeprevir therapy, when it occurred alongside major resistance mutations at position 155 and/or position 168.[29] Other major NS3 RAMs occurred at codons 36, 54, 55, 168, and 170, which is consistent with previous observations.[1-6, 9, 30]. **A small subset of five subjects had Q80K plus one other major RAM in NS3**.

In summary, we found a high prevalence of Q80K in HCV-1a carriers with and without HIV accessing care in two regions of the UK, thus complementing estimates from elsewhere in Europe. While deep sequencing increased detection of Q80K, mutants missed by Sanger sequencing occurred at frequency ≤0.5% and detection seemed a likely technical artefact. There was evidence of a high degree of interspersing of UK sequences with sequences from elsewhere in Europe and North America, although the phylogeny indicated that most transmission events occurred in the UK. Investigation of full viral genomes will elucidate HCV-1a transmission networks and gain data to inform control strategies.

Table 1. Prevalence of NS3 resistance-associated mutations (RAMs) in treatment-naïve HCV-1a carriers **according to modality of detection (Sanger or Illumina) and frequency of the mutant as determined by Illumina**[a]

RAMs	Sanger/Illumina >10% n (%)	Illumina 1-10% n (%)
Total tested	238 (100)	178 (100)
36 M	1 (0.4)	2 (1.1)
36 L	5 (2.1)	0 (0)
54 S	9 (3.8)	0 (0)
55 A	10 (4.2)	0 (0)
80 K	44 (18.5)	0 (0)
80 L	1 (0.4)	4 (2.2)
80 R	0 (0)	3 (1.7)
117 H	0 (0)	1 (0.6)
168 E	1 (0.4)	0 (0)
170 A	1 (0.4)	0 (0)
170 T	0 (0)	2 (1.1)
174 S	97 (40.8)	10 (5.6)

[a]RAMs were classified according to the geno2pheno mutation list (March 2015 version) with the addition of Q80L (reference 11). Bold indicates major mutations for genotype 1/1a.

277 **Figure 1**. Phylogenetic analysis of HCV-1a NS3 sequences from the United Kingdom, where
278 green indicates sequences from the North-West and yellow indicates sequences from the
279 South-East of England. **The global reference dataset was derived from Los Alamos HCV**
280 **database (March 2015; see supplementary file for accession numbers)** and comprises
281 sequences from the rest of Europe (in red) and North America (in blue). Nodes with bootstrap
282 support values ≥90% are indicated with an asterisk.
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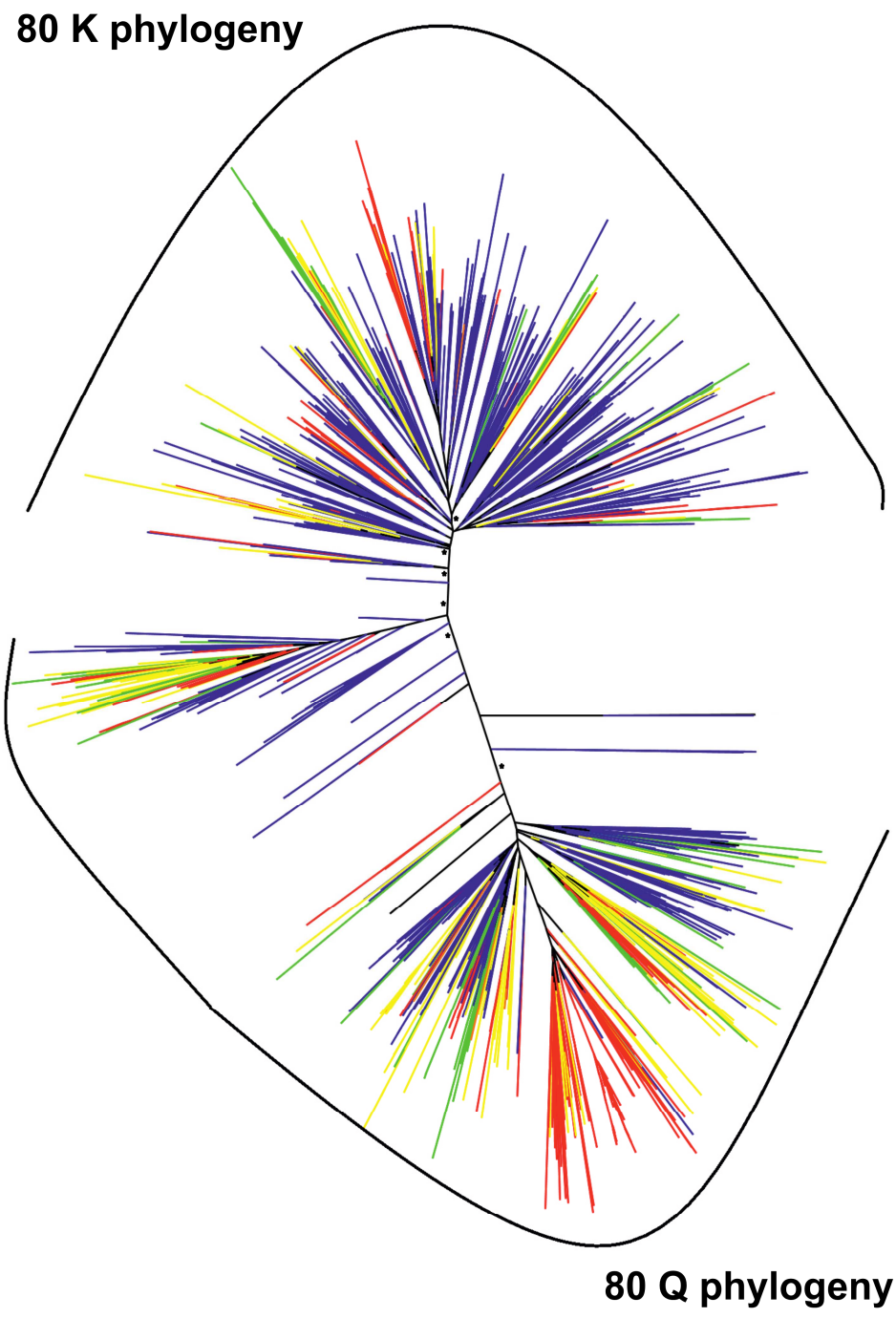

284 **Figure 2**. A HCV transmission cluster in the South-East of England, comprising 20 HCV-1a
285 NS3 sequences (in yellow). Sequences from the rest of Europe and North America are shown
286 in red and blue respectively. Nodes with bootstrap support values ≥90% are indicated with an
287 asterisk.

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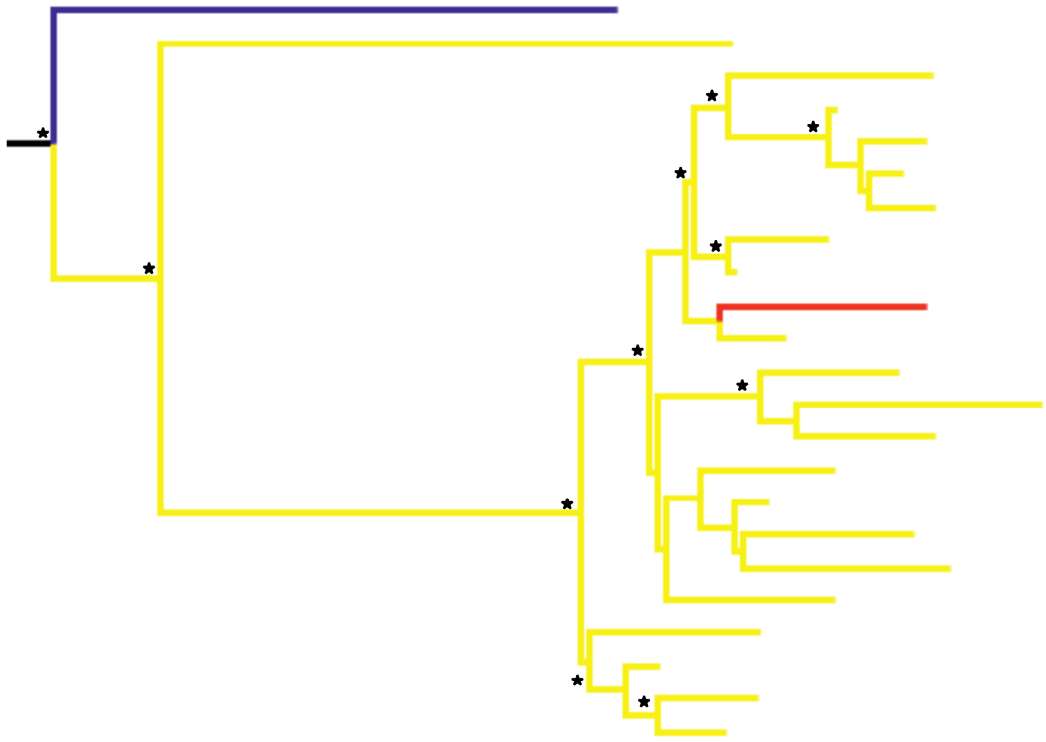

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References

1 Cento V, Mirabelli C, Salpini R, et al. Hcv genotypes are differently prone to the development of resistance to linear and macrocyclic protease inhibitors. *PloS one*. 2012; **7**: e39652.

2 Bae A, Sun SC, Qi X, et al. Susceptibility of treatment-naive hepatitis c virus (HCV) clinical isolates to hcv protease inhibitors. *Antimicrobial Agents and Chemotherapy*. 2010; **54**: 5288-5297.

3 Bartels DJ, Sullivan JC, Zhang EZ, et al. Hepatitis c virus variants with decreased sensitivity to direct-acting antivirals (daas) were rarely observed in daa-naive patients prior to treatment. *Journal of Virology*. 2013; **87**: 1544-1553.

4 Berger KL, Triki I, Cartier M, et al. Baseline hepatitis c virus (hcv) ns3 polymorphisms and their impact on treatment response in clinical studies of the hcv ns3 protease inhibitor faldaprevir. *Antimicrobial Agents and Chemotherapy*. 2014; **58**: 698-705.

5 Kuntzen T, Timm J, Berical A, et al. Naturally occurring dominant resistance mutations to hepatitis c virus protease and polymerase inhibitors in treatment-naive patients. *Hepatology*. 2008; **48**: 1769-1778.

6 Leggewie M, Sreenu VB, Abdelrahman T, et al. Natural ns3 resistance polymorphisms occur frequently prior to treatment in hiv-positive patients with acute hepatitis c. *AIDS*. 2013; **27**: 2485-2488.

7 Morel V, Duverlie G, Brochot E. Patients eligible for treatment with simeprevir in a french center. *Journal of Clinical Virology*. 2014; **61**: 149-151.

8 Sarrazin C, Lathouwers E, Peeters M, et al. Prevalence of the hepatitis c virus ns3 polymorphism q80k in genotype 1 patients in the european region. *Antiviral Research*. 2015; **116C**: 10-16.

9 Vicenti I, Rosi A, Saladini F, et al. Naturally occurring hepatitis c virus (HCV) ns3/4a protease inhibitor resistance-related mutations in HCV genotype 1-infected subjects in italy. *Journal of Antimicrobial Chemotherapy*. 2012; **67**: 984-987.

10 Jabara CB, Hu F, Mollan KR, et al. Hepatitis c virus (HCV) NS3 sequence diversity and antiviral resistance-associated variant frequency in HCV/HIV coinfection. *Antimicrobial Agents and Chemotherapy*. 2014; **58**: 6079-6092.

11 Lenz O, Verbinnen T, Lin TI, et al. In vitro resistance profile of the hepatitis c virus ns3/4a protease inhibitor tmc435. *Antimicrobial Agents and Chemotherapy*. 2010; **54**: 1878-1887.

12 Forns X, Lawitz E, Zeuzem S, et al. Simeprevir with peginterferon and ribavirin leads to high rates of svr in patients with hcv genotype 1 who relapsed after previous therapy: A phase 3 trial. *Gastroenterology*. 2014; **146**: 1669-1679 e1663.

13 Jacobson IM, Dore GJ, Foster GR, et al. Simeprevir with pegylated interferon alfa 2a plus ribavirin in treatment-naive patients with chronic hepatitis c virus genotype 1

infection (quest-1): A phase 3, randomised, double-blind, placebo-controlled trial. *Lancet*. 2014; **384**: 403-413.

14 Manns M, Marcellin P, Poordad F, et al. Simeprevir with pegylated interferon alfa 2a or 2b plus ribavirin in treatment-naive patients with chronic hepatitis c virus genotype 1 infection (quest-2): A randomised, double-blind, placebo-controlled phase 3 trial. *Lancet*. 2014; **384**: 414-426.

15 Simeprevir summary of product characteristics. Available at: Http://www.Ema.Europa.Eu/docs/en_gb/document_library/epar_-_product_information/human/002777/wc500167867.Pdf. (accessed 3 march 2015).

16 M. M. Cutadapt removes adapter sequences from high-throughput sequencing reads. . *EMBnet J* 2011; **17**: 10-12.

17 Verbist BM, Thys K, Reumers J, et al. Virvarseq: A low-frequency virus variant detection pipeline for illumina sequencing using adaptive base-calling accuracy filtering. *Bioinformatics*. 2015; **31**: 94-101.

18 Dierynck I, Thys K, Ghys A, et al. Deep-sequencing analysis of the gene encoding the hepatitis c virus nonstructural 3-4a protease confirms a low prevalence of telaprevir-resistant variants at baseline and the end of the realize study. *Journal of Infectious Diseases*. 2014; **210**: 1871-1880.

19 Beloukas A, Magiorkinis E, Magiorkinis G, et al. Assessment of phylogenetic sensitivity for reconstructing hiv-1 epidemiological relationships. *Virus Res*. 2012; **166**: 54-60.

20 Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of phyml 3.0. *Systematic Biology*. 2010; **59**: 307-321.

21 Price MN, Dehal PS, Arkin AP. Fasttree 2--approximately maximum-likelihood trees for large alignments. *PloS One*. 2010; **5**: e9490.

22 Gianella S, Delport W, Pacold ME, et al. Detection of minority resistance during early hiv-1 infection: Natural variation and spurious detection rather than transmission and evolution of multiple viral variants. *Journal of Virology*. 2011; **85**: 8359-8367.

23 McCloskey RM, Liang RH, Joy JB, et al. Global origin and transmission of hepatitis c virus nonstructural protein 3 q80k polymorphism. *Journal of Infectious Diseases*. 2014.

24 Pickett BE, Striker R, Lefkowitz EJ. Evidence for separation of hcv subtype 1a into two distinct clades. *Journal of Viral Hepatitis*. 2011; **18**: 608-618.

25 **Lawitz E, Sulkowski MS, Ghalib R, et al. Simeprevir plus sofosbuvir, with or without ribavirin, to treat chronic infection with hepatitis c virus genotype 1 in non-responders to pegylated interferon and ribavirin and treatment-naive patients: The cosmos randomised study. *Lancet*. 2014; 384: 1756-1765.**

26 Lawitz E, Matusow G, DeJesus E. A phase 3, open-label, single-arm study to evaluate the efficacy and safety of 12 weeks of simeprevir (smv) plus sofosbuvir (sof) in treatment-naive or experienced patients with chronic HCV genotype 1 (HCV-1) infection and cirrhosis: Optimist-2. *50th Annual Meeting of the European Association for the Study of the Liver*. Vienna, Austria - April 22-26, 2015.

27 McPhee F, Friborg J, Levine S, et al. Resistance analysis of the hepatitis c virus NS3 protease inhibitor asunaprevir. *Antimicrobial Agents and Chemotherapy*. 2012; **56**: 3670-3681.

28 Pilot-Matias T, Tripathi R, Cohen D, et al. In vitro and in vivo antiviral activity and resistance profile of the hepatitis c virus NS3/4a protease inhibitor abt-450. *Antimicrobial Agents and Chemotherapy*. 2015; **59**: 988-997.

29 Lenz O, Verbinnen T, Fevery B, et al. Virology analyses of HCV isolates from genotype 1-infected patients treated with simeprevir plus peginterferon/ribavirin in phase iib/iii studies. *Journal of Hepatology*. 2014.

30 Akuta N, Suzuki F, Sezaki H, et al. Evolution of simeprevir-resistant variants over time by ultra-deep sequencing in hcv genotype 1b. *Journal of Medical Virology*. 2014; **86**: 1314-1322.

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AF011752
AF011753
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AY695437
AY956463
AY956464
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